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ENGRAILED 2 (EN2)

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14. ABSTRACT Autism Spectrum Disorder (ASD) risk is likely due to both genetic susceptibility and non-genetic environmental factors. The environment can influence gene expression by epigenetic differences. Our previous research demonstrated the homeobox transcription factors, ENGRAILED 2 (EN2) is genetically associated with ASD. The ASD-associated variant is also functional, increasing levels both in vitro and in vivo. Human post-mortem studies also demonstrate increased EN2 levels. We then hypothesized that increased levels could also be due to epigenetic changes since EN2 is flanked by 6 significant CpG islands. To investigate this question we treated two human neuronal cell lines with the methylation inhibitor, AZA, and the methyl donor, SAM. AZA treated cell lines resulted in increased EN2 mRNA levels while SAM decreased expression. Preliminary bisulfite sequencing indicates changes in EN2 mRNA levels are correlated with differences in DNA methylation. The same methylation differences are observed in the above post-mortem samples, suggesting epigenetic changes may also contribute to the increase of EN2 observed in these samples.					
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INTRODUCTION: Risk for ASD is due to both genetic susceptibility and non-genetic factors that can influence gene expression through epigenetic dysregulation. Our previous genetic findings demonstrated the homeobox transcription factor, *ENGRAILED 2* (*EN2*), is significantly and consistently associated with ASD (Gharani et al., 2004; Benayed et al., 2005). Six other groups have also reported association for *EN2* with ASD in their datasets (Petit et al., 1995; Wang et al., 2007; Brune et al., 2007; Yang et al., 2008; Sen et al., 2010; Yang et al., 2010). We observed an intronic haplotype (*rs1861972-rs1861973* A-C) is over-transmitted from parents to affected individuals. The same haplotype is under-represented in unaffected siblings (518 families, $P=.00000035$). Moreover, the ASD-associated A-C haplotype is functional, causing increased expression both in primary neuronal cultures and transgenic mice. Finally increased *EN2* mRNA levels have been observed in ASD cerebellar post-mortem samples compared to age and sex matched controls (90 samples; $P=.0038$). These results suggest elevated *EN2* levels may be correlated with increased ASD risk. Thus we decided to investigate whether epigenetic differences could also result in increased expression. This possibility is supported by the presence of 6 significant CpG islands flanking *EN2*. The goal of the W81XWH-09-1-0286 DoD award was two-fold: i) to investigate if *EN2* is epigenetically regulated by CpG methylation and ii) if hypomethylation of these flanking CpG islands contribute to increased *EN2* mRNA levels in the above post-mortem samples

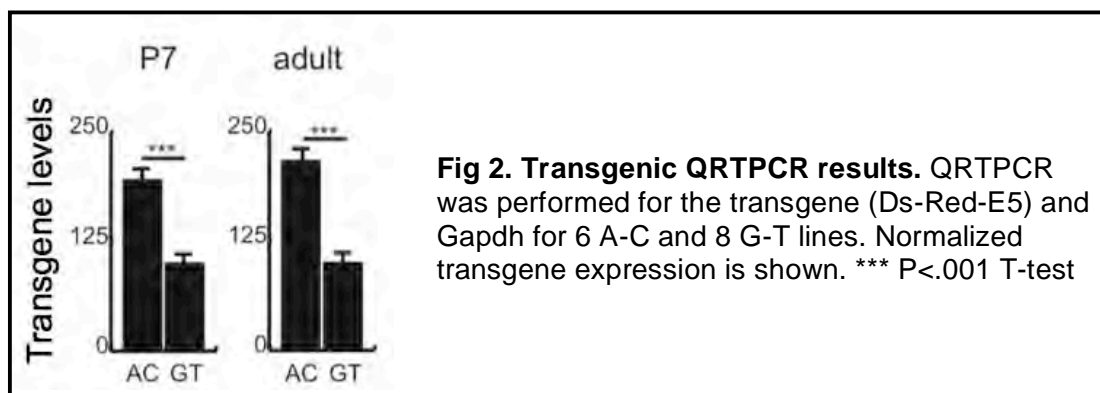
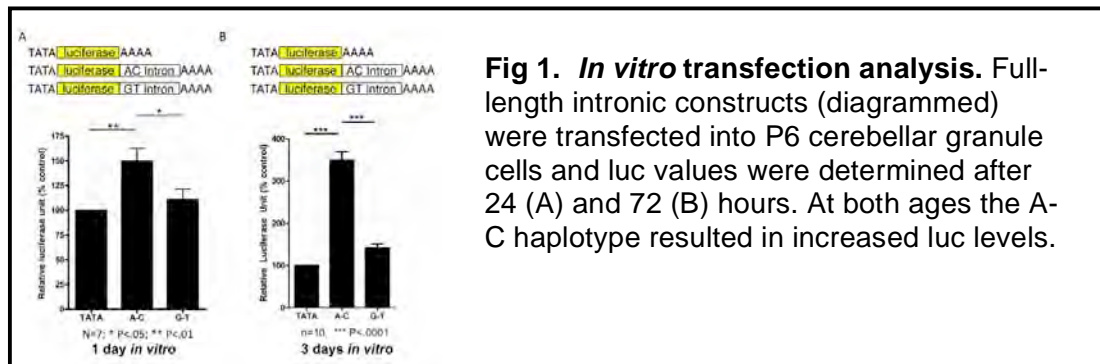
BODY: To accomplish these goals we needed to do the following experiments:

- 1) Obtain human neuronal cell lines that express *EN2* at high levels according to bioinformatic websites (Aim 1)
- 2) Verify cell line *EN2* expression by QRT-PCR (Aim 1)
- 3) Treat the cell lines with increasing concentrations of 5-aza-2'-deoxycytidine (AZA), a methylation inhibitor (Aim 1)
- 4) Treat the cell lines with the methyl donor, S-adenosylmethionine (SAM) (Aim 1)
- 5) Measure *EN2* mRNA levels by QRT-PCR in treated and untreated cell lines (Aim 1)
- 6) Perform bisulfite sequencing on treated cell lines to verify expression differences are due to differential CpG methylation (Aim 1)
- 7) Perform bisulfite sequencing on post-mortem samples to investigate if increased *EN2* mRNA levels are correlated with CpG hypomethylation (Aim 2)

We made the following progress:

We selected two human neuronal cell lines (Daoy and SH-SY5Y) based upon high *EN2* expression in publicly available databases (Gene Expression Omnibus-GEO; Cancer Genome Anatomy Project). In addition both cell lines are generated from medulloblastoma tumors. These childhood tumors typically originate from cerebellar granule neurons, making them more biologically relevant than other human neuronal cell lines for the following two reasons. One, cerebellar granule neurons can be isolated and grown in culture. Using this cell culture system, we have demonstrated the *EN2* ASD associated A-C haplotype is functional in these neurons (Fig 1). Two, we have generated transgenic mice for the A-C haplotype and the other common haplotype (G-T) which is inherited more frequently in unaffected siblings. In the post-natal and adult cerebellum, endogenous *En2* and the transgene are expressed specifically in cerebellar granule cells. At P6 and in the adult, the A-C haplotype results in increased levels of the transgene (Fig 2). Thus the ASD-associated A-C haplotype is functional *in vitro* and *in*

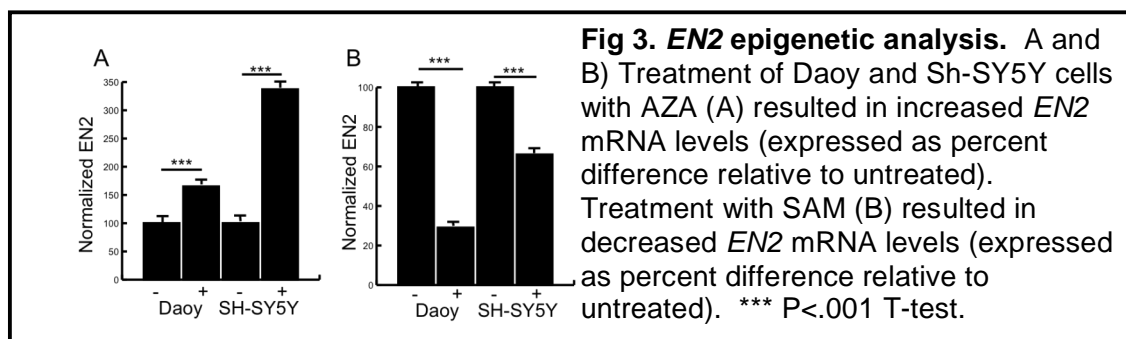
vivo in cerebellar granule cells. For these reasons, we decided to test the epigenetic regulation of *EN2* in medulloblastoma cell lines.



The Daoy and SH-SY5Y cell lines were obtained from ATCC and grown in my lab using established protocols. The lines were then expanded, aliquots were frozen for future use, and RNA was isolated. Taqman QRTPCR primers were obtained and *EN2* levels were quantified, and normalized against GAPDH. *EN2* is expressed at significant levels in both cell lines.

Because the ASD associated A-C haplotype can also affect *EN2* expression, we genotyped both cell lines for *rs1861972* and *rs1861973* using established approaches. Both lines have an A-C/G-T genotype.

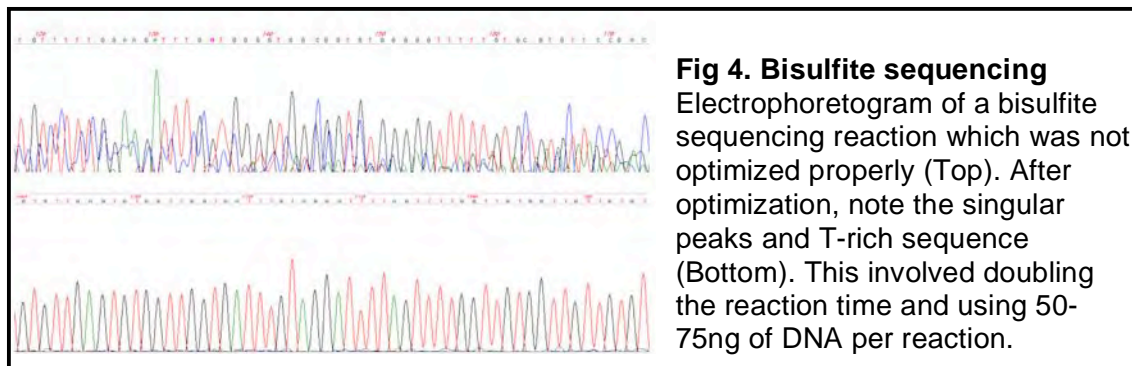
To test our hypothesis that *EN2* is epigenetically regulated by differential methylation, we treated both cell lines with increasing concentrations of AZA (0, 1, 5, 10uM) and SAM (0, .25, 1, 2 mM). If methylation of the flanking *EN2* CpG islands inhibits transcription, we would expect treating with the methyl donor SAM to decrease *EN2* mRNA levels. Conversely, AZA, a methylation inhibitor should result in increased *EN2* mRNA levels.



Both cell lines were then treated with SAM or AZA at the above concentrations for 72hrs. The cells were harvested, RNA isolated and normalized *EN2* mRNA levels were quantified. For the AZA treatment, a significant increase in *EN2* mRNA levels was observed for both cell lines. For the SAM experiments, *EN2* mRNA levels were decreased significantly (Fig 3-10uM AZA; SAM 2mM). These results are consistent with *EN2* being epigenetically regulated by differential methylation.

Next to determine if the effect on *EN2* mRNA levels is direct and due to differences in methylation, bisulfite sequencing was conducted. Our analysis was initially focused on the promoter CpG island, which spans ~5.6k and comprises 404 CpGs. We estimated this region could be amplified using ~12 primer pairs.

However, numerous problems were encountered in the PCR amplification of the bisulfite treated genomic DNA. We believe these issues are due to the very high CG content (58.1%) of the sequence. To circumvent these technical difficulties, several parameters were optimized, which are summarized below.

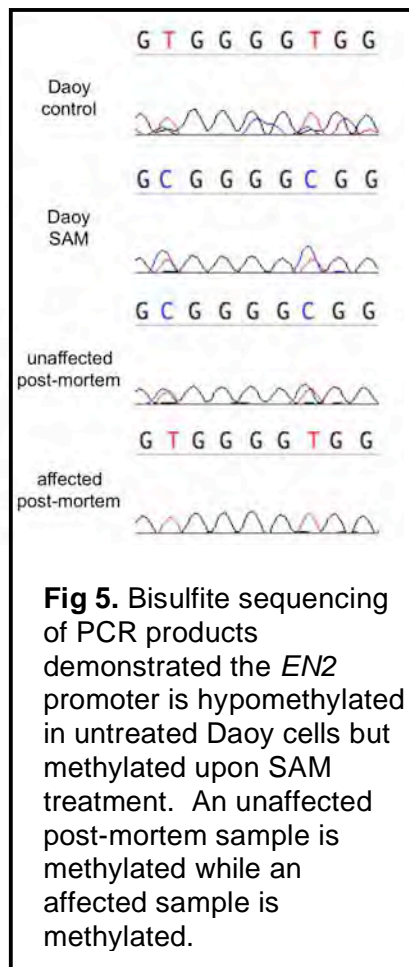


First, different protocols were tested for treating the genomic DNA with bisulfite. In total four kits from various manufacturers were tried and we discovered the two with a modified version of bisulfite worked the best. Typically the genomic DNA is incubated with bisulfite overnight. However the modified bisulfite allowed us to reduce the incubation time to ~1hr. Once these kits were successfully identified, we then titrated the incubation time and the amount of bisulfite. We discovered incubating 50-75ng of genomic DNA with bisulfite for 2hrs generated the best results (Fig 4).

Next, primer selection for the genomic PCR was optimized. We employed 3 different programs: Methyl-seq, Methprimer, and MacVector. Methyl-seq generated primers that consistently failed while Methprimer and MacVector generated some useful primers. After trying a number of parameters, the best solution for consistent results was the selection of primers by Methprimer. These primers were then further optimized by adding nucleotides to increase the T_m as well as shifting or extending the sequence so the primer was anchored in a G or C nucleotide.

We then had to decide whether to submit the PCR products directly for sequencing or subcloning the amplicons and sequencing multiple positive clones. We opted for sequencing each PCR product directly because it would better evaluate whether a particular CpG dinucleotide was partially methylated. The degree of methylation could also be approximated by the size of the peaks on the electrophoretogram. However, the sequencing of the PCR product consistently failed. We then worked with the sequencing

company to further optimize the sequencing and found increasing the amount of submitted DNA resulted in better results.



We then decided to use nested PCR to generate more of the PCR product. These changes resulted in the successful sequencing of the PCR product as long as the previous PCR primers were degraded by an ExoSap reaction.

In summary these experiments were more challenging than originally thought. Nevertheless we were successful and have now only recently started to bisulfite sequencing the promoter, focusing on the region spanning the transcriptional start site. We observe 6 methylated CpGs with SAM treatment and 0 methylated CpGs in AZA treated cells (Fig 5). Additional bisulfite sequencing is ongoing but these data are consistent with *EN2* being epigenetically regulated. The above results support the epigenetic regulation of *EN2* due to differential methylation.

Next we investigated whether methylation in the post-mortem sample is affected and correlated with changes in *EN2* mRNA levels. We have bisulfite sequenced 5 post-mortem samples for the promoter amplicon. In affected individuals none of the CpG dinucleotides were methylated. In unaffected individuals, 6 CpGs were methylated. These CpGs are the same dinucleotides methylated after SAM treatment *in vitro* (Fig 5). Bisulfite sequencing is currently being conducted on additional amplicons from more post-mortem samples.

These results are consistent with *EN2* being epigenetically regulated by differential methylation. In addition, hypomethylation of the flanking CpG islands may contribute to elevated *EN2* mRNA levels observed in affected post-mortem samples. These results suggest non-genetic factors during CNS development could affect *EN2* levels through epigenetic regulation. To identify these non-genetic factors and when and where during CNS development they exert effects, an *EN2* knock-in mouse is being generated where ~75kb of the mouse gene is being replaced with the human sequence. This sequence includes the 6 flanking CpG islands.

KEY RESEARCH ACCOMPLISHMENTS

- Treating two cell lines with AZA increased *EN2* mRNA levels
- Treating both cell lines with SAM decreased *EN2* mRNA levels

- Preliminary bisulfite sequencing of the AZA and SAM treated cell lines indicates hypomethylation is correlated with increased *EN2* mRNA levels while hypermethylation decreases expression.
- Preliminary bisulfite sequencing of post-mortem samples suggests differential methylation also contributes to increased *EN2* levels in affected post-mortem samples.

REPORTABLE OUTCOMES

The above data was presented in part at the following talks and published abstracts:

Talks: Mighty Mouse Symposium, Rider University, November 2009
Autism Workshop, UMDNJ-RWJ, June 2010

Abstracts: Choi J, Kamdar S, Matteson PG, Brzustowicz L, Millonig JH. *In vitro* and *in vivo* functional characterization of the *ENGRAILED 2* ASD-associated haplotype. The 9th International Meeting for Autism research (IMFAR), Philadelphia, PA June, 2010 (poster presentation)

Grants: The above data was also used to obtain addition funding from the NJ Governor's Council for Autism Research (6/01/10-4/30/12; \$469,550 total costs)

CONCLUSION

The data generated during the W81XWH-09-1-0286 DoD grant is consistent with *EN2* being epigenetically regulated by differential methylation, and suggest both genetic and non-genetic factors function in concert to increase *EN2* levels in individuals with ASD